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Host investment into symbiosis varies among genotypes of the legume *Acmispon strigosus*, but host sanctions are uniform

Camille E. Wendlandt[1], John U. Regus[2], Kelsey A. Gano-Cohen[3], Amanda C. Hollowell[2,4], Kenjiro W. Quides[2], Jonathan Y. Lyu[2], Eunice S. Adinata[2] and Joel L. Sachs[1,2,3,4]

[1]Department of Botany & Plant Sciences, University of California, Riverside, CA 92521, USA; [2]Department of Evolution, Ecology & Organismal Biology, University of California, Riverside, CA 92521, USA; [3]Department of Microbiology & Plant Pathology, University of California, Riverside, CA 92521, USA; [4]Institute for Integrative Genome Biology, University of California, Riverside, CA 92521, USA

Author for correspondence:
Joel L. Sachs
Tel: +1 951 827 6357
Email: joels@ucr.edu

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Summary

- Efficient host control predicts the extirpation of ineffective symbionts, but they are nonetheless widespread in nature. We tested three hypotheses for the maintenance of symbiotic variation in rhizobia that associate with a native legume: partner mismatch between host and symbiont, such that symbiont effectiveness varies with host genotype; resource satiation, whereby extrinsic sources of nutrients relax host control; and variation in host control among host genotypes.
- We inoculated *Acmispon strigosus* from six populations with three *Bradyrhizobium* strains that vary in symbiotic effectiveness on sympatric hosts. We measured proxies of host and symbiont fitness in single- and co-inoculations under fertilization treatments of zero added nitrogen (N) and near-growth-saturating N. We examined two components of host control: 'host investment' into nodule size during single- and co-inoculations, and 'host sanctions' against less effective strains during co-inoculations.
- The *Bradyrhizobium* strains displayed conserved growth effects on hosts, and host control did not decline under experimental fertilization. Host sanctions were robust in all hosts, but host lines from different populations varied significantly in measures of host investment in both single- and co-inoculation experiments.
- Variation in host investment could promote variation in symbiotic effectiveness and prevent the extinction of ineffective *Bradyrhizobium* from natural populations.

Introduction

Plants can exhibit elegant host control traits that preferentially select beneficial over ineffective symbionts. For instance, yuccas and fig trees abort developing fruits that are overburdened by eggs of their specialized pollinators and preferentially allocate resources into fruits serviced by more effective pollinators (Pellmyr & Huth, 1994; Jandér *et al.*, 2012; Jandér & Herre, 2016). Barrel medics degrade arbuscules of mycorrhizas that do not deliver phosphorus (P) (Javot *et al.*, 2007), and soybeans reduce growth of intracellular rhizobia that fail to fix nitrogen (N) (Kiers *et al.*, 2003). Provided there are no other sources of selection on symbiotic services, host control traits are predicted to impose directional selection on symbiotic partners, reducing variation in the symbiotic services provided and favoring the fixation of beneficial genotypes (Fig. 1a; Denison, 2000; West *et al.*, 2002a,b; Foster & Kokko, 2006; Foster & Wenseleers, 2006). In nature, however, plant-associated symbionts commonly vary from beneficial to ineffective (Johnson *et al.*, 1997; Moawad *et al.*, 1998; Burdon *et al.*, 1999; Chen *et al.*, 2002; Carú *et al.*, 2003; Markham, 2008; Bromfield *et al.*, 2010; Sachs *et al.*, 2010a; Otero *et al.*, 2011; Granada *et al.*, 2014). Thus, there is a key gap in our knowledge: models predicting that host control traits purify populations of ineffective symbionts fail to explain the maintenance of variation in these diverse symbioses. An emerging framework predicts that both genetic and environmental sources of variation in host control traits can promote the maintenance of symbiont variation, but tests of this framework remain scant.

Three main hypotheses can be identified for the maintenance of symbiont variation in interactions between plant hosts and microbes (Fig. 1). Under the partner mismatch hypothesis, symbionts that are ineffective or mediocre on one host genotype are maintained in a population because they are beneficial (i.e. effective) on other hosts due to specificity interactions (Bever, 1999; Burdon *et al.*, 1999; Heath & Tiffin, 2007; Heath, 2010; Barrett *et al.*, 2012). Even if all host genotypes exert host control traits over ineffective partners, partner mismatch would cause ineffective symbiont genotypes to be punished in a host-specific manner (Fig. 1b). Intuitively, partner mismatch is more likely when hosts

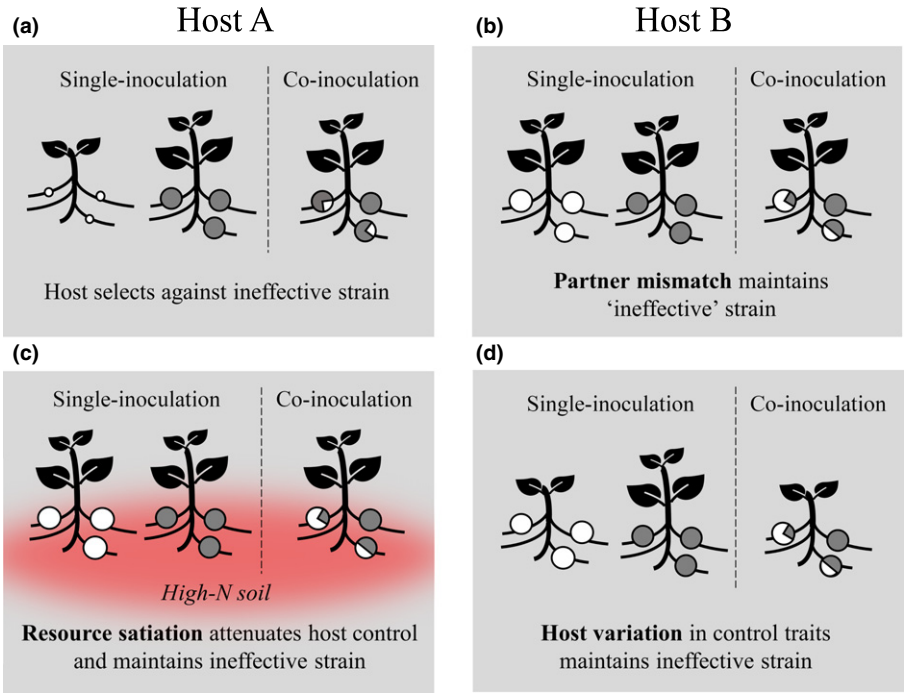

Fig. 1 Three hypotheses for how variation in symbiotic effectiveness of rhizobia is maintained. (a) Mutualism theory and empirical studies predict that hosts will select against an ineffective strain (white) relative to an effective strain (grey) by forming relatively small nodules during single inoculations and reducing nodule occupancy during co-inoculations. Over time, host-mediated selection is predicted to drive the ineffective strain extinct and reduce variation in symbiotic effectiveness among rhizobia, inconsistent with the high variation in effectiveness seen in soils worldwide. (b–d) Scenarios that reduce the fitness differential between the ineffective and effective strains by reducing their difference in nodule size (single inoculations) or nodule occupancy (co-inoculations). (b) Under the 'partner mismatch' scenario, the 'ineffective' strain is symbiotically effective on a different host genotype (Host B), enabling the 'ineffective' strain to form large nodules during single inoculations and achieve high nodule occupancy during co-inoculations. (c) Under the 'resource satiation' scenario, Host A relaxes host control traits when its nitrogen needs are met by the soil; although Host A does not benefit from the ineffective strain, relaxed host control enables the ineffective strain to form large nodules during single inoculations and achieve high nodule occupancy during co-inoculations. (d) Under the 'host variation' scenario, the ineffective strain encounters a different host genotype (Host B) that fails to exert host control traits; although Host B does not benefit from the ineffective strain, it allows the ineffective strain to form large nodules during single inoculations and achieve high nodule occupancy during co-inoculations.

interact with symbionts whose typical host is a different species (Thrall *et al.*, 2000). However, partner mismatch has also been observed among genotypes of the same host species (Burdon *et al.*, 1999; Heath, 2010). Thus, partner mismatch could be an important mechanism for maintaining variation in symbiont effectiveness at local scales.

Symbiont variation can also be maintained if host control itself varies. Variation in host control could occur physiologically (within a host genotype, depending on the external environment) or genetically (among host genotypes). In resource mutualisms like the legume–rhizobia symbiosis, physiological attenuation of host control traits could occur when plants encounter extrinsic sources of nutrients normally offered by symbionts. Under the resource satiation hypothesis, plants are predicted to switch to cheap mineral sources of nutrients when they are plentiful (Bronstein, 1994; West *et al.*, 2002b; Thrall *et al.*, 2007; Shantz *et al.*, 2016) and to downregulate costly pathways involved in symbiosis, including host control (Fig. 1c). Therefore, spatial variation in soil nutrients could generate variation in host control over ineffective symbionts. There is evidence that resource satiation can lead hosts to downregulate symbiosis pathways in some systems, for instance when N fertilization causes legumes to form fewer root nodules with rhizobia (Streeter & Wong, 1988; Saturno *et al.*, 2017 and references therein). But other studies have found mixed effects (Heath *et al.*, 2010) or no effects of resource satiation on host control traits (Kiers *et al.*, 2006; Regus *et al.*, 2014; Grillo *et al.*, 2016). Thus, the role of resource satiation in the maintenance of variation in symbiont effectiveness requires further study.

The host variation hypothesis predicts that host control traits vary among host genotypes such that some host genotypes are more efficient at host control than others (Fig. 1d). Steidinger & Bever (2014) offered one model of how host genotypes differing in host control traits could coexist in a population through negative plant–soil feedbacks. Briefly, host genotypes with strong host control traits ('discriminators') are predicted to drive down the frequency of ineffective symbionts until only effective symbionts are regularly encountered. If host control is costly for hosts, genotypes with weak host control traits ('givers') are predicted to outperform discriminators when effective symbionts are abundant. However, givers would act as a refuge for ineffective symbionts and allow their frequency to rise, shifting selection to favor discriminator hosts. This dynamic equilibrium among host control strategies would also maintain populations of both ineffective

and effective symbionts. This model and others (Foster & Kokko, 2006) suggest that alternative host strategies could be driven by costs of host control. Some empirical studies have failed to find evidence of host control (Marco *et al.*, 2009, 2015; Gubry-Rangin *et al.*, 2010; Grillo *et al.*, 2016), consistent with the host variation hypothesis, but only a few studies have examined genetic variation of host control across populations of a species (Heath & Tiffin, 2009; Simonsen & Stinchcombe, 2014; Haney *et al.*, 2015). A common theme in the partner mismatch, resource satiation, and host variation hypotheses is that context dependency of either symbiont effectiveness or host control traits could maintain variation in symbiont effectiveness.

Host control can be measured by host sanctions against ineffective symbionts and host investment into symbiotic structures. Host sanctions lead to differences in symbiont relative fitness when hosts are infected by multiple symbiont genotypes, such that the most effective symbiont achieves the greatest relative fitness (Denison, 2000; Kiers *et al.*, 2003; Sachs *et al.*, 2004). Host investment into symbiotic structures can also be a measure of host control if the resources that flow to symbionts affects symbiont fitness (e.g. previous work has found correlations between nodule size and rhizobia per nodule for individual rhizobial genotypes; Kiers *et al.*, 2003; Heath & Tiffin, 2007). Co-inoculations permit the measurement of both host sanctions and host investment, but single inoculations only permit the measurement of host investment, and inferring host control from single inoculations requires comparing host investment into symbiont genotypes that vary in effectiveness on the same host genotype. This approach allows researchers to minimize the number of factors explaining symbiont fitness, but the no-choice design can generate autocorrelation of host and symbiont fitness components due to fitness feedbacks (Oono *et al.*, 2009, 2011; Kiers *et al.*, 2013). Therefore, performing parallel single and co-inoculations generates a more thorough understanding of host control.

Here, we investigate mechanisms maintaining variation in effectiveness of *Bradyrhizobium* symbionts in a metapopulation of *Acmispon strigosus* hosts (formerly *Lotus strigosus*). *A. strigosus* is an annual legume native to the southwestern United States that associates with N fixing, root-nodulating rhizobia in the genus *Bradyrhizobium* (Sachs *et al.*, 2009). Like many legume species, *A. strigosus* initiates nodules with compatible rhizobial genotypes soon after germination. *A. strigosus* nodules grow rapidly and the *Bradyrhizobium* within nodules proliferate (Sachs *et al.*, 2010a). The nodules begin to senesce as the plant flowers and begins pod filling, a stage at which nodule rhizobia are released back into the soil. At a well studied population at Bodega Marine Reserve (BMR) in northern California, *A. strigosus* are nodulated by *Bradyrhizobium* strains that range from highly effective (e.g., *c.* 6-fold growth improvement of inoculated sympatric hosts relative to uninoculated controls) to ineffective (e.g., no growth improvement of inoculated sympatric hosts; Sachs *et al.*, 2010a). This striking variation is consistent with other surveys of symbiont effectiveness, which have uncovered both effective and ineffective symbionts (Burdon *et al.*, 1999; Rangin *et al.*, 2008; Bromfield *et al.*, 2010; Ehinger *et al.*, 2014). *A. strigosus* hosts from BMR also demonstrate efficient host control when inoculated with sympatric *Bradyrhizobium* that vary in effectiveness, forming nodules of reduced size with ineffective strains (i.e. reduced host investment; Regus *et al.*, 2015), and showing reduced *in planta* abundance of ineffective strains during co-inoculations with effective strains (i.e. host sanctions; Sachs *et al.*, 2010b; Regus *et al.*, 2014). The co-occurrence of ineffective symbionts and robust host control traits in the *Acmispon–Bradyrhizobium* system makes it powerful for testing hypotheses about the maintenance of variation in symbiont effectiveness.

We inoculated three *Bradyrhizobium* strains onto *A. strigosus* hosts from six populations and grew plants with and without mineral sources of N. The host populations were sampled from across a 10-fold range of soil N levels (2–20 ppm mineral N; Regus *et al.*, 2017). We sampled *A. strigosus* more deeply across populations than within populations (one to two seed sets from each population) to maximize the chance of sampling different genotypes from this species. We tested our hypotheses first in a single inoculation design, in which each host was inoculated with a clonal culture of each strain, and also in a co-inoculation design, where each host was inoculated simultaneously with all three strains. In the single inoculation experiment, we measured host benefits (relative growth, ^{15}N discrimination, and 'symbiotic efficiency' *sensu* Oono & Denison, 2010) and investment (mean nodule size) to infer symbiont effectiveness and host control, respectively. In the co-inoculation experiment, we quantified sanctions (the proportion of nodules occupied by the most effective strain, i.e. 'nodule occupancy') and investment (mean nodule size) to infer host control. We also compared plant relative growth from co-inoculation with the mean benefits from all single-inoculation treatments to examine whether host control is associated with increased host benefits relative to the null expectation from single-inoculations (Heath & Tiffin, 2007). Under the partner mismatch hypothesis, we predicted that the effectiveness of each *Bradyrhizobium* strain would vary among *A. strigosus* hosts, such that the least effective strain on one host would be the intermediate or most effective strain on another host. Under the resource satiation hypothesis, we predicted that hosts would display weaker host control in fertilized than unfertilized conditions. Under the host variation hypothesis, we predicted that hosts would exhibit genetic differences in host control traits. Our work contributes to an emerging theoretical framework to explain the maintenance of variation in populations of microbial mutualists (Sachs *et al.*, 2011a; Heath & Stinchcombe, 2013; Steidinger & Bever, 2014, 2016; Bever, 2015; Pahua *et al.*, 2018).

Materials and Methods

Bradyrhizobium strains

Bradyrhizobium isolates 05LoS21R6.43 (strain #18), 05LoS3.3 (strain #38), and 05LoS24R3.28 (strain #2) were isolated in 2005 from *A. strigosus* root surfaces or nodules at Bodega Marine Reserve ('BMR,' Sonoma Co., CA, USA; Sachs *et al.*, 2009). Strains #18 and #38 provide different amounts of fixed N to sympatric BMR hosts, increasing shoot mass by *c.* 6-fold and 4-fold respectively, compared with uninoculated control hosts,

whereas strain #2 forms nodules on sympatric hosts but does not enhance growth for these hosts (i.e. ineffective; Sachs *et al.*, 2010a; Regus *et al.*, 2015). In a survey of 1292 *Bradyrhizobium* isolates across California, including the six field sites from which we sourced *A. strigosus* seeds for this experiment, the *glnII_recA* haplotypes corresponding to strains #18, #38 and #2 were only recovered at BMR (Hollowell *et al.*, 2016a). This finding suggests that *A. strigosus* hosts from other field sites are not coevolved with these strains and improves the chance of uncovering partner mismatch in this system.

Acmispon strigosus host lines

A. strigosus seeds were collected from six field sites from the northern range (1 site; BMR) and southern range (five sites) of this species in California between 2005 and 2012 (Calflora). Field sites varied in soil N levels (Regus *et al.*, 2014, 2017): soil mineral N was low (2–4 ppm) at Anza-Borrego Desert State Park ('Anz') and Bodega Marine Reserve ('BMR'), intermediate (*c.* 7 ppm) at Griffith Park ('Gri') and Burns-Pinyon Ridge Reserve near Yucca Valley ('Yuc'), and high (11–20 ppm) at Bernard Field Station of the Claremont Colleges ('Cla') and University of California, Riverside ('UCR').

We raised plants from wild seeds in a glasshouse sprayed with insecticide to eliminate insect pollination, allowed plants to self, and collected seeds from individual plants to generate inbred lines. We selected two inbred seed sets derived from different wild seed ancestors per field site, but we used wild mixed seeds from Gri because there was poor seed production from those plants (Supporting Information Table S1). Inbred host lines are now referred to as Anz03, Anz11, BMR04, BMR07, Cla06, Cla10, UCR03, UCR10, Yuc02, and Yuc03, and the wild Gri seeds are referred to as Gri01.

We assessed genetic divergence among *A. strigosus* lines by sequencing the *nuclear ribosomal Internal Transcribed Spacer* (*nrITS*) and the nuclear gene *Cyclin Nucleotide Gated Channel 5* (*CNGC5*; Table S2). These loci have been used to resolve phylogenetic relationships within the legume tribe Loteae (*nrITS*, Allan & Porter, 2000) and the legume genus *Medicago* (*CNGC5*, Maureira-Butler *et al.*, 2008). For each inbred host line, we sequenced loci from at least two progeny of the wild seed ancestor defining the host line. To genotype the wild Gri01 seed set, we sequenced loci from at least eight plants grown from wild seeds. All analyzed nucleotide positions had at least 2x sequencing coverage. Sequence gaps were eliminated before pairwise distance analysis in MEGA7 (Kumar *et al.*, 2016). Sequences are deposited in GenBank under accession numbers KX449152–KX449173.

Inoculation experiments

We raised axenic *A. strigosus* seedlings in sterilized quartzite sand in Ray-Leach SC10 containers (Stuewe & Sons, Corvallis, OR, USA) following published protocols (Sachs *et al.*, 2009). Plants with true leaves were hardened for 8 d in the glasshouse until inoculation on 4 March 2014. We grew *Bradyrhizobium* strains on modified arabinose gluconate (MAG) agar plates, washed cells off plates into liquid MAG, quantified cell titers by colorimetry, pelleted the cells, and resuspended them in sterile ddH_2O to generate inocula of 1×10^8 cells ml^{-1} (Sachs *et al.*, 2009). Plants were inoculated with 5 ml of clonal *Bradyrhizobium* cultures (single inoculation experiment), 5 ml of a mixture comprising equal concentrations of each culture (co-inoculation experiment) or 5 ml sterile ddH_2O as a control (both experiments). Fertilization treatments consisted of weekly applications of 5 ml N-free Jensen's solution ('unfertilized' plants) or 5 ml Jensen's with 0.5 g l^{-1} $K^{15}NO_3$ (2% ^{15}N by weight; 'fertilized' plants), beginning 4 days before inoculation (Sachs *et al.*, 2009).

The single inoculation experiment included 288 plants (six host populations × two host lines × four inoculation treatments × two fertilization treatments × three plant replicates). The experiment was blocked by host line (Anz11, BMR07, Cla10, UCR10 and Yuc02 were placed on one glasshouse bench and Anz03, BMR04, Cla06, UCR03 and Yuc03 were placed on a second glasshouse bench, with Gri01 split evenly between the two benches). Size-matched plants from each host line were randomly assigned to each fertilization and inoculation treatment. Plant positions were randomized within blocks. The co-inoculation experiment included 240 plants (six host populations × two host lines × two inoculation treatments × two fertilization treatments × five plant replicates). Larger seedlings were used for the co-inoculation experiment so that competing *Bradyrhizobium* strains would have access to the larger root systems of these plants. The co-inoculation experiment was split into two blocks and plants were size-matched and randomly assigned to fertilization and inoculation treatments, as in the single inoculation experiment.

Plant harvest and nodule culturing

The single- and co-inoculation experiments were harvested 51–57 days post-inoculation (dpi) and 48–55 dpi, respectively. Plants were removed from pots, washed free of sand, and dissected into root, shoot, and nodule portions. Nodules were counted and photographed against graph paper to measure nodule area (ImageJ). Roots, shoots, and nodules not used for culturing were oven dried (> 4 days, 60°C) and weighed. An empirically generated nodule area to mass equation was used to correct per-plant nodule dry masses for nodules removed for culturing:

$$\text{Nodule dry mass (mg)} = \frac{\text{Nodule area (mm}^2) - 0.9097853}{5.5258444}$$

Leaf tissue from singly inoculated plants was assayed for ^{15}N content (one plant replicate per treatment; 96 samples; UC Davis Stable Isotope Facility).

Nodules for culturing were chosen from the upper and lower 50% of the nodule size distribution on the plant, avoiding senescent (green or brown) nodules. Nodules were surface sterilized with bleach, rinsed, crushed, and spread onto two replicate MAG-agar plates in 10^{-3} and 10^{-5} dilutions (single inoculation experiment) or onto three replicate plates to

generate isolated colonies (co-inoculation experiment; Sachs *et al.*, 2009). From the single inoculation experiment, we cultured two nodules from one plant replicate of each host line and treatment (144 nodules total) and calculated number of rhizobial cells per nodule from at least two plates containing 3–800 colonies. From the co-inoculation experiment, we cultured four nodules from two plant replicates of each host line and treatment (192 nodules total). An average of 102 colonies per nodule were sub-cultured onto three separate MAG-agar plates containing: (1) 125 μg ml^{-1} streptomycin; (2) 100 μg ml^{-1} gentamycin; and (3) no antibiotic (positive control). Strain #2 is resistant to gentamycin and streptomycin, #18 is resistant to gentamycin, and #38 is sensitive to both. Colonies were scored after 4 days of growth at 29°C. Colonies with ambiguous scores were sub-cultured again, and colonies with persistent ambiguous scores (0.4% of all colonies streaked) were excluded from calculations of nodule occupancy.

Estimating host benefits and host control over symbiosis

We estimated net host benefits from symbiosis as relative growth:

$$\text{Relative growth} = \frac{\text{Total plant (root + shoot) DM}_{I+}}{\text{Total plant DM}_{I-}},$$

where DM = dry mass in mg, I+ = inoculated, and I− = uninoculated. Relative growth greater than one indicates growth benefit from inoculation. To estimate host benefits in the context of their level of investment into nodules, we calculated symbiotic efficiency (*sensu* Oono and Denison, 2010):

$$\text{Symbiotic efficiency} = \frac{\text{Total plant DM}_{I+} - \text{Total plant DM}_{I-}}{\text{Total nodule DM}}.$$

For unfertilized singly-inoculated plants, we measured ^{15}N discrimination (a proxy for nitrogen fixation), which is deviation from the atmospheric ^{15}N atom percentage due to isotopic fractionation by nitrogenase (i.e., δ^{15}N; Unkovich, *et al.*, 2008). For fertilized singly-inoculated plants, we calculated percent nitrogen derived from the atmosphere (percent Ndfa) from δ^{15}N values of size-matched plants:

$$\%\text{Ndfa} = 100 * \frac{\delta^{15}N_{F+I-} - \delta^{15}N_{F+I+}}{\delta^{15}N_{F+I-} - \delta^{15}N_{F-I+}},$$

where F+ = fertilized and F− = unfertilized. During single- and co-inoculations, we estimated host control using mean nodule size (total nodule dry mass divided by total nodule number). We also examined total nodule dry mass and total nodule number separately to understand how those traits contributed to variation in nodule size. During co-inoculations, we estimated host control using nodule occupancy of the most effective strain (identified during single-inoculations). We estimated both 'inclusive' nodule occupancy (counting all nodules in which the most effective strain was found, including co-infected nodules) and 'exclusive' nodule occupancy (counting only nodules singly-infected by the most effective strain).

Data analysis

We used linear regressions to test whether mean nodule size significantly predicted number of rhizobial cells per nodule for each strain during single inoculations. We used general linear mixed models (GLMMs) to test estimates of host benefit (relative growth, symbiotic efficiency, δ^{15}N, and percent Ndfa) and host control (mean nodule size, total nodule dry mass, and total nodule number) for effects of host population, strain, fertilization, and interactions among those effects. Block was included as a random effect in all models. We removed non-significant interaction terms if this reduced AICc values by at least two units (Table S3). Significant differences among treatments were assessed using pairwise *t*-tests (Tukey's HSD) of least squares means, with significant interaction terms pre-empting significant main effects. Dependent variables were log-transformed if necessary to improve normality. We used the binomial test to evaluate whether nodule occupancy of the most effective strain deviated from the null expectation of 33%. To understand whether host control was associated with increased host benefits relative to the null expectation from single inoculations (Heath & Tiffin, 2007), we tested relative growth from co-inoculations against mean relative growth from single inoculations for each host/fertilizer treatment using one-sample *t*-tests (single inoculation means were calculated from *c.* 18 plants: three strains × two blocks × three plant replicates). Statistical analyses were performed in JMP PRO 13.0.0 (SAS Institute Inc., Cary, NC, USA) and Microsoft EXCEL (2016).

Data availability

Raw date for this article has been submitted to Dryad and is available at doi: 10.5061/dryad.fq15r87.

Results

Genotyping *A. strigosus* host lines

Sympatric host lines within BMR, Cla, UCR, and Yuc populations were identical at *nrITS* and *CNGC5* loci, but Anz03 and Anz11 differed at both loci. Host lines from different populations generally differed at both loci, but UCR and BMR hosts could not be differentiated using these loci (Table S2).

Nodulation of *Bradyrhizobium* strains on *Acmispon* host lines

In the single inoculation experiment, strain #18 formed nodules on all but two inoculated plants (Anz11 and Cla10 host lines) and strain #38 formed nodules on all inoculated plants. Strain #2 formed nodules on most inoculated plants but failed to nodulate five of six Anz11 plants (the one nodulated plant bore only a

single nodule) and one additional plant (Cla10). Inoculated plants that failed to form nodules were excluded from subsequent analyses. All co-inoculated plants formed nodules. There were no nodules on uninoculated plants.

Benefits of symbiosis during single inoculations

Genetic effects on host benefits Strain #18 was highly effective and #2 was ineffective on all unfertilized hosts, consistent with previous studies (Sachs *et al.*, 2010a). Of the three strains, #18 produced the greatest plant relative growth, symbiotic efficiency, and ^{15}N discrimination ($\delta^{15}N = -1.6‰$), consistent with high rates of N fixation (Figs 2, S1; Table 1). Strain #38 was intermediate in ^{15}N discrimination ($\delta^{15}N = -0.43‰$), and strain #2 ($\delta^{15}N = 1.29‰$) did not significantly differ from uninoculated plants ($\delta^{15}N = 1.76‰$). Strain #38 was intermediate in effectiveness to strains #2 and #18 for BMR and Gri hosts but equally as effective as #18 for Anz, Cla, UCR, and Yuc hosts (Fig. 2). Of the five hosts, Cla hosts achieved the greatest relative growth and symbiotic efficiency from the effective strains (#18, #38) and Gri hosts achieved the least, although the remaining hosts did not significantly differ from these two extremes (Figs 2, S1).

Fertilization effects on host benefits Fertilization reduced plant relative growth with the effective strains and reduced the difference in their effectiveness (#18: 83% Ndfa; #38: 69% Ndfa), although both remained more effective than #2 (0.4% Ndfa; Fig. 2; Table S4). Fertilization reduced symbiotic efficiency for all hosts (Fig. S1).

Host control over symbiosis during single inoculations

Regression analysis of number of rhizobia per nodule against nodule size The number of rhizobia per nodule had a positive relationship with nodule size for strains #2 (adj. $R^2 = 0.37$, $P = 0.0002$, slope $= 2.0 \times 10^7$ cells mm^{-2} nodule) and #18 (adj. $R^2 = 0.12$, $P = 0.0126$, slope $= 3.2 \times 10^6$ cells mm^{-2} nodule), but not for intermediate strain #38 (adj. $R^2 = 0.06$, $P = 0.0890$, slope $= 7.8 \times 10^5$ cells mm^{-2} nodule; Fig. S2). The steeper regression line for strain #2 vs #18 corroborates previous

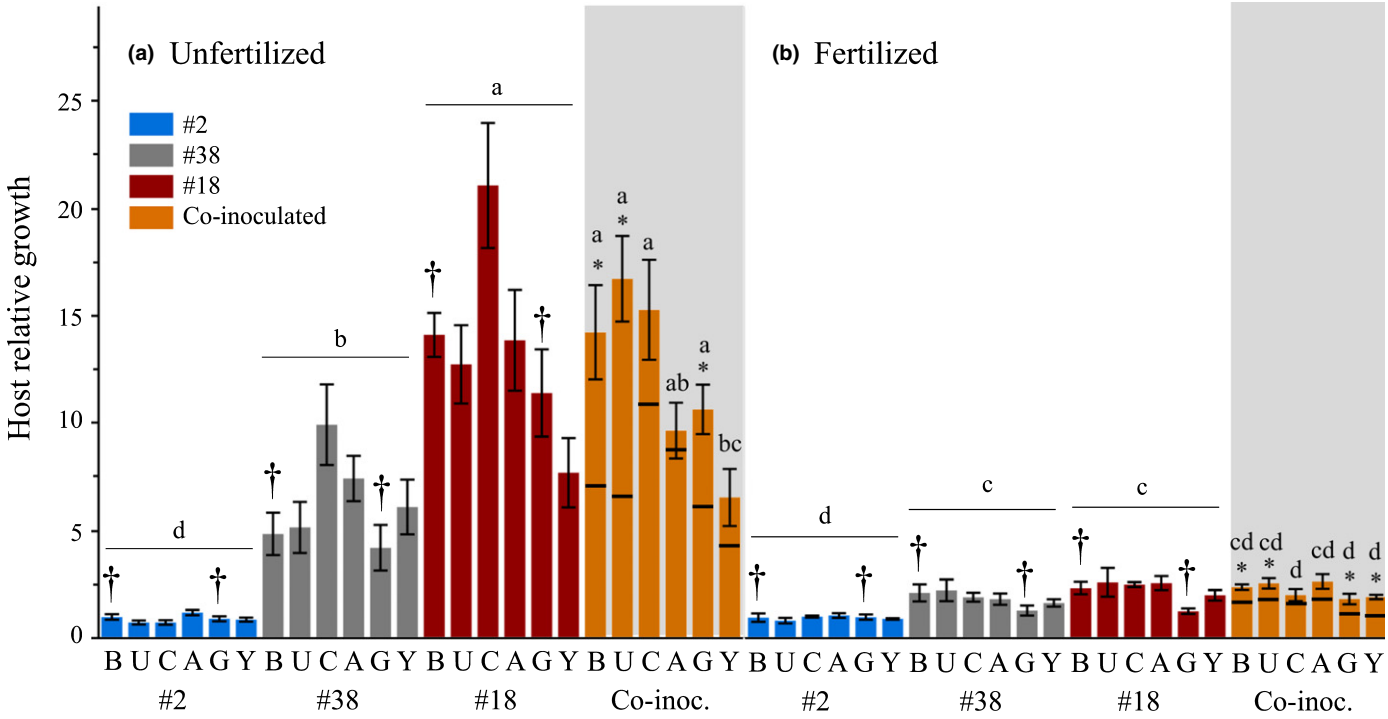

Fig. 2 Test of the partner mismatch hypothesis based on plant relative growth of *Acmispon strigosus* from different populations in (a) unfertilized and (b) fertilized conditions. Relative growth was calculated as total plant dry mass (roots + shoots) of the inoculated plant divided by the total plant dry mass of its size-matched uninoculated control plant. Relative growth greater than one indicates growth benefit from symbiosis. Statistics were performed separately for singly and co-inoculated plants. For singly inoculated plants, different letters above strain treatments indicate significant differences among strain and fertilization treatments (strain × fertilization effect; Table 1). Daggers above a host population indicate that plant relative growth differed significantly among all three strains (#18 > #38 > #2); populations not marked with a dagger had significant growth differences only for strain #2 vs the other two strains (population × strain effect; Table 1). For co-inoculated plants, different letters indicate significant differences among population and fertilization treatments (population × fertilization effect; Table 1). Bold horizontal bars indicate the mean relative growth combining all three single-inoculation treatments for each host population in each fertilization treatment. Asterisks indicate that relative growth of co-inoculated plants significantly differed from the mean of single-inoculation treatments in a one-sample *t*-test ($P > 0.05$). B, BMR (Bodega Marine Reserve); U, UCR (University of California, Riverside); C, Cla (Bernard Field Station of the Claremont Colleges); A, Anz (Anza-Borrego Desert State Park); G, Gri (Griffith Park); Y, Yuc (Burns-Pinyon Ridge Reserve near Yucca Valley). Bars represent ± 1 standard error (SE).

Table 1 Models testing the partner mismatch hypothesis for singly inoculated *Acmispon strigosus* and variation in symbiotic host benefits for co-inoculated *A. strigosus*

	Log_{10} (Plant relative growth)		Symbiotic efficiency		[A]Log_{10} ($\delta^{15}N+3$)		[B]Percent Ndfa	
	$n = 208$, Adj. $R^2 = 0.85$		$n = 207$; Adj. $R^2 = 0.59$		$n = 43$; Adj. $R^2 = 0.67$		$n = 34$; Adj. $R^2 = 0.95$	
Single inoculation	df	*F*	df	*F*	df	*F*	df	*F*
Host population	5, 186.1	5.5442***	5, 197.1	2.7692*	5, 33.02	1.8395	5, 25	0.7117
Strain	2, 186.1	313.372***	2, 197.1	123.0521***	3, 33.17	27.7131***	2, 25.1	341.3825***
Fertilization	1, 186	275.2759***	1, 197	27.6379***	.	.	.	.
Pop × Strain	10, 186	2.1776*	.	.	.	.	.	.
Strain × Fertilization	2, 186	89.168***	.	.	.	.	.	.
Co-inoculation	Log_{10} (Plant relative growth)		Symbiotic efficiency					
	$n = 119$; Adj. $R^2 = 0.72$		$n = 119$; Adj. $R^2 = 0.21$					
	df	*F*	df	*F*				
Host population	5, 106	5.0272***	5, 106	2.4095*				
Fertilization	1, 106	267.674***	1, 106	16.5919***				
Pop × Fertilization	5, 106	3.3398**	5, 106	2.1596				

[A]The model used only unfertilized plants and also included uninoculated control plants such that the 'strain' effect included the control treatment; one outlier 5.5 SD above the mean (plant 25) was excluded; we added 3 to all raw values before log-transformation (this was the smallest integer that made all values positive).
[B]The model used only fertilized plants.
*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

findings that strain #2 achieves high population sizes in small nodules (Sachs *et al.*, 2010a). Thus, we used mean nodule size as a proxy of host control for strain #2 and #18, understanding that strain #2 had greater within-nodule population density than strain #18.

Genetic effects on host investment Anz, BMR, and UCR hosts invested in rhizobia in a benefits-dependent way, making larger nodules for #18 than #2, with #38 intermediate (Fig. 3; Table 2). Cla, Gri, and Yuc hosts invested in rhizobia irrespective of benefits, showing no significant difference in nodule size among strains. Among hosts inoculated with strain #18, UCR and BMR hosts made the largest nodules and Yuc hosts made the smallest. Among hosts inoculated with strain #2, Gri hosts made the largest nodules and UCR hosts made the smallest. Hosts did not vary in nodule size for strain #38. Total nodule dry mass was greater for the effective strains (#18, #38) than ineffective strain #2 for hosts from each population (Fig. S3a; Table 2). For unfertilized plants, hosts from Cla, Gri, and Yuc formed more nodules with the effective strains (#18, #38) than ineffective strain #2, but hosts from Anz, BMR, and UCR did not significantly differ in total nodule number among strains (Fig. S3b; Table 2).

Fertilization effects on host investment Fertilization reduced nodule size for strain #2 but did not affect nodule size for strains #18 or #38 (Fig. 3; Table 2). Fertilization increased total nodule dry mass for all strains, although strains #18 and #38 still had greater total nodule dry mass than ineffective strain #2 in fertilized conditions. Fertilization increased total nodule dry mass for all hosts except Yuc (Fig. S3a; Table 2). Fertilized Yuc hosts also formed more nodules with the effective strains (#18, #38) than ineffective strain #2 while the remaining hosts did not differ in total nodule number among strains under fertilization (Fig. S3b; Table 2).

Benefits of symbiosis during co-inoculations

Co-inoculated Yuc hosts had lower relative growth than other hosts in unfertilized conditions (Fig. 2; Table 1). Relative growth of co-inoculated BMR, Gri, and UCR hosts exceeded their single inoculation means, whereas Anz and Cla hosts had similar growth in both experiments. These patterns were not altered by fertilization. Relative growth of unfertilized Yuc hosts did not differ from the single inoculation mean, but fertilized Yuc hosts gained more growth from co-inoculation than the single inoculation mean (Fig. 2). Fertilization reduced the relative growth of all co-inoculated plants and erased the differences among hosts seen in unfertilized conditions. Symbiotic efficiency was greatest for Gri hosts and least for BMR hosts, although hosts from other populations did not significantly differ from those extremes (Fig. S1; Table 1). Fertilization reduced symbiotic efficiency.

Host control over symbiosis during co-inoculations

Host sanctions As the mean total nodule number on co-inoculated plants ranged from 40 (unfertilized plants) to 57 (fertilized plants), our nodule occupancy assays tested 7–10% of the nodules on selected plants. The most effective strain (#18)

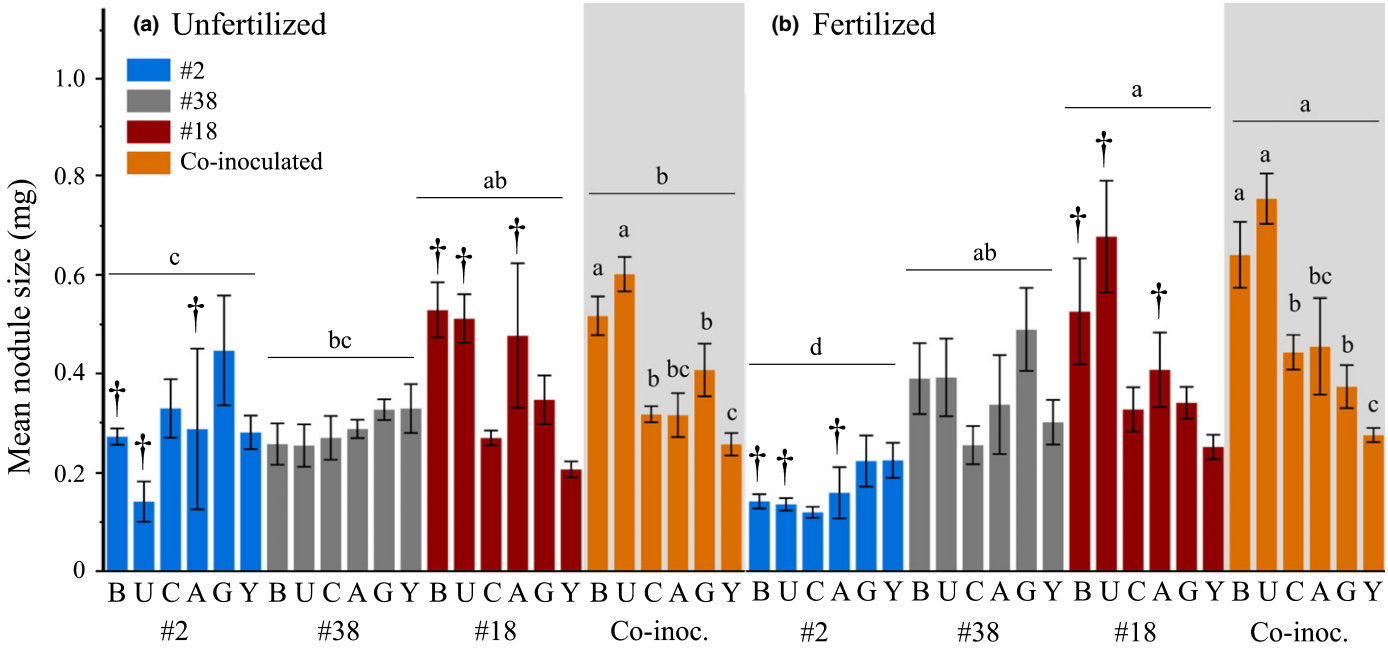

Fig. 3 Test of the resource satiation and host variation hypotheses using mean nodule size of *Acmispon strigosus* from different populations in (a) unfertilized and (b) fertilized conditions. Mean nodule size was calculated as total nodule dry mass divided by total nodule number. Statistics were performed separately for singly inoculated and co-inoculated plants. For singly inoculated plants, different letters above strain treatments indicate significant differences among strain and fertilization treatments (strain × fertilization effect; Table 2). Daggers indicate host populations that produced significantly larger nodules with strain #18 than #2; populations not marked with a dagger did not significantly differ in nodule size for those strains (population × strain effect; Table 2). For co-inoculated plants, different letters above populations indicate significant differences among populations, whereas different letters above fertilization treatments indicate significant differences between fertilization treatments. B, BMR (Bodega Marine Reserve); U, UCR (University of California, Riverside); C, Cla (Bernard Field Station of the Claremont Colleges); A, Anz (Anza-Borrego Desert State Park); G, Gri (Griffith Park); Y, Yuc (Burns-Pinyon Ridge Reserve near Yucca Valley). Bars represent ± 1 standard error (SE).

dominated the majority of nodules on all tested hosts (Fig. 4). Of 19 312 colonies scored from nodules, 96.4%, 2.6%, and 1.0% were identified as strains #18, #38 and #2, respectively. Strain #38 was recovered from hosts of all six populations in the unfertilized treatment and from BMR, Anz, and Yuc hosts in the fertilized treatment. Strain #2 was recovered from UCR and Cla hosts in the unfertilized treatment and from UCR and Anz hosts in the fertilized treatment. Strains #18, #38 and #2 were identified in 170, 19, and six nodules, respectively, from the 177 nodules successfully sub-cultured from co-inoculated plants. Seventeen nodules were co-infected by more than one strain. For each host population × fertilization treatment combination, strain #18 was identified in nodules more often than expected by chance under a null nodule occupancy of 33% (binomial test, all $P < 0.0001$ for inclusive nodule occupancy and all $P < 0.0016$ for exclusive nodule occupancy).

Host investment Mean nodule size varied significantly among hosts from different populations: UCR and BMR hosts produced the largest nodules and Yuc hosts produced the smallest, with the remaining populations intermediate (Fig. 3, Table 2). There was little variation among hosts for total nodule dry mass, but total nodule number was significantly greater for Yuc hosts than BMR, UCR, or Cla hosts (Fig. S3, Table 2). Fertilization increased mean nodule size, total nodule dry mass and total nodule number.

Discussion

To understand how variation in symbiont effectiveness is maintained, we tested for three kinds of refugia that could protect ineffective symbionts from host-mediated purifying selection. We found no evidence of partner mismatch in our panel of three *Bradyrhizobium* strains and six population sources of *Acmispon strigosus*. Neither did we find evidence that resource satiation relaxed host control over the ineffective symbiont. However, hosts from different populations differed in host control traits, consistent with the host variation hypothesis.

Host variation hypothesis

Empirical evidence of host control exists for several legume species, including soybean (*Glycine max*; Kiers *et al.*, 2003), alfalfa and pea (*Medicago sativa* and *Pisum sativum*; Oono *et al.*, 2011), *Medicago lupulina* (Simonsen & Stinchcombe, 2014), and *A. strigosus* (Sachs *et al.*, 2010b). Here, we tested for host control as host sanctions during co-inoculations and host investment into nodule size during both co-inoculations and single inoculations. Host genotypes from all six *A. strigosus* populations showed evidence of robust host sanctions, corroborating previous studies using mixed seed sources from BMR (Sachs *et al.*, 2010b; Regus *et al.*, 2014). By contrast, we found genetic variation for host investment into symbionts. Since

Table 2 Models testing the resource satiation and host variation hypotheses for singly inoculated and co-inoculated *Acmispon strigosus*

	Log_{10} (Mean nodule size)		Log_{10} (Total nodule dry mass)		Log_{10} (Total nodule number)	
	$n = 207$; Adj. $R^2 = 0.40$		$n = 207$; Adj. $R^2 = 0.74$		$n = 208$; Adj. $R^2 = 0.64$	
Single inoculation	df	*F*	df	*F*	df	*F*
Host population	5, 185.1	2.6963*	5, 179	3.8622**	5, 171.1	4.4561***
Strain	2, 185.1	36.8121***	2, 180.6	193.8363***	2, 171.1	52.2038***
Fertilization	1, 185	0.9426	1, 180	167.0683***	1, 171	139.025***
Pop × Strain	10, 185.1	4.9066***	10, 179.6	2.4856**	10, 171.1	4.9277***
Pop × Fertilization	.	.	5, 180.1	3.5277**	5, 171	3.0261*
Strain × Fertilization	2, 185	8.3954***	2, 180.3	6.0356**	2, 171	13.2344***
Pop × Strain × Fert	.	.	.	.	10, 171	2.1854*
	Log_{10} (Mean nodule size)		Total nodule dry mass		Log_{10} (Total nodule number)	
	$n = 120$; Adj. $R^2 = 0.46$		$n = 120$; Adj. $R^2 = 0.57$		$n = 120$; Adj. $R^2 = 0.46$	
Co-inoculation	df	*F*	df	*F*	df	*F*
Host population	5, 112	20.0028***	5, 107	2.6878*	5, 112	15.2086***
Fertilization	1, 112	7.3474**	1, 107	158.7695***	1, 112	30.4258***
Pop × Fertilization	.	.	5, 107	1.4717	.	.

*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

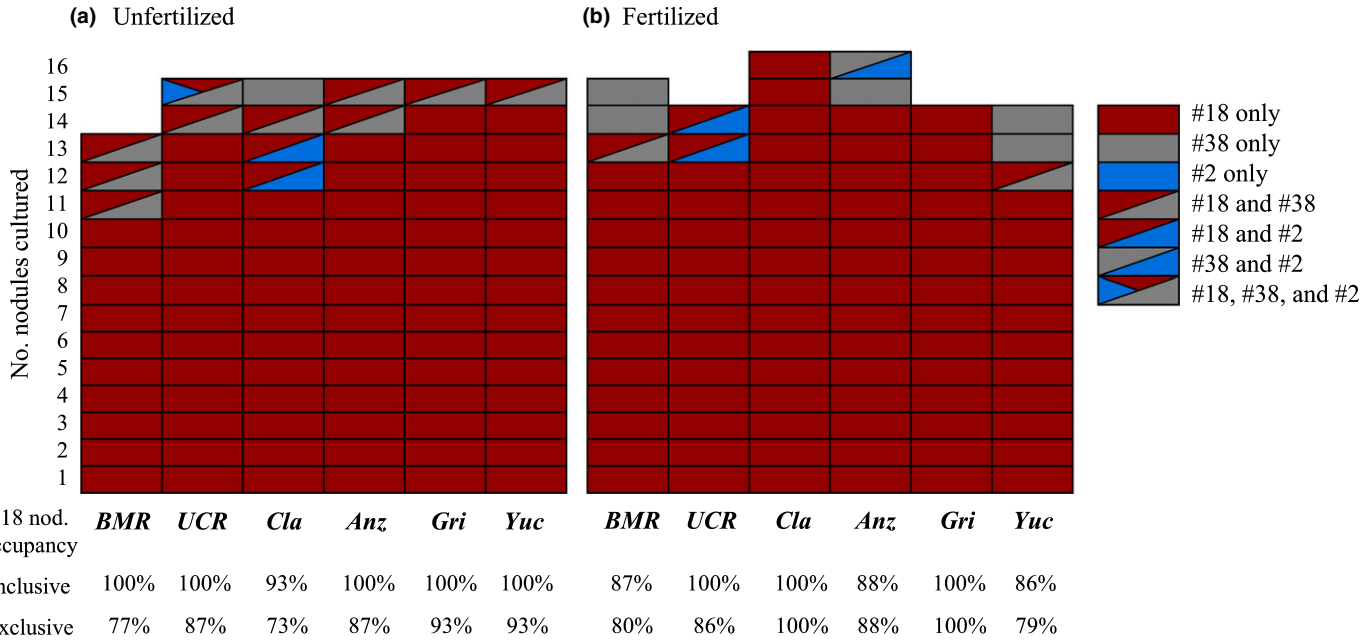

Fig. 4 Test of the resource satiation and host variation hypotheses using frequency of co-inoculated *Bradyrhizobium* strains in cultured nodules of *Acmispon strigosus* in (a) unfertilized and (b) fertilized conditions. Up to 16 nodules were cultured from plants of each host population (four nodules × two plant replicates × two host lines). The strain occupancy of each nodule was determined by sub-culturing isolated colonies onto selective media. Nodule occupancy by effective strain #18 was calculated as the percentage of nodules that contained #18, whether or not other strains were also present (inclusive), and the percentage of nodules that contained only #18, without any other strains present (exclusive). BMR, Bodega Marine Reserve; UCR, University of California, Riverside; Cla, Bernard Field Station of the Claremont Colleges; Anz, Anza-Borrego Desert State Park; Gri, Griffith Park; Yuc, Burns-Pinyon Ridge Reserve near Yucca Valley.

population genetic structure of rhizobia can cause hosts to encounter different subsets of symbiont genotypes in nature, host variation in investment could affect symbiont relative fitness in situations where hosts encounter just one or a few strains, or one strain in large numerical excess to others (McInnes *et al.*, 2004; Hollowell *et al.*, 2016a). Therefore, variation in host control operating at the level of host investment into nodules, but not at the level of host sanctions, could help maintain variation in symbiont effectiveness in the *Acmispon–Bradyrhizobium* system.

Strain #18 was generally the most effective strain for all hosts, inconsistent with the partner mismatch hypothesis, and the dominance of strain #18 in nodules of co-inoculated plants (with the near absence of less effective strains) is consistent with robust host sanctions in plants from all host populations. However, strain #38 approached the effectiveness of strain #18 on some hosts, particularly in fertilized conditions. If symbiont nodule occupancy were strictly a function of sanctions acting on strain effectiveness, we would expect to see more evidence of strain #38 in nodules of co-inoculated plants. Our data support the hypothesis that *in planta* symbiont fitness is a joint function of symbiont competitive ability and sanctions acting on symbiont effectiveness, consistent with other published data: Amarger (1981) showed that similarly effective strains co-inoculated onto *Medicago sativa* were not recovered from nodules in their inoculation ratio, but showed differential competitive ability *in planta*, and similar results exist for *Medicago truncatula* (Grillo *et al.*, 2016). We found evidence that strain #38 had lower cell titers in nodules of singly inoculated plants than other strains (Fig. S2), and previous work found that strain #38 had lower *in vitro* doubling rates than strains #2 or #18 (Sachs *et al.*, 2010b), possibly explaining its failure to significantly occupy nodules of co-inoculated plants. The dominance of effective strain #18 over ineffective strain #2 in this study, however, is consistent with sanctions, as previously reported (Sachs *et al.*, 2010b). The conservation of host sanctions across genotypes from different host populations and fertilization treatments suggests that this component of host control is fixed in *A. strigosus*.

We found significant variation among hosts from different populations in the degree to which they invested into nodules harboring the most effective strain (Fig. 3). Differences in symbiotic efficiency among hosts were modest compared with differences among strains and fertilization treatments (Fig. S1), suggesting that different hosts experience similar benefits from symbiosis per unit nodule mass. However, we found that plants from three host populations (UCR, BMR, Anz) showed 'scaled investment' during single inoculations by increasing the size of nodules as benefits from symbiosis increased, whereas plants from Cla, Gri, and Yuc populations showed 'unscaled investment' (nodule size did not significantly change with changing benefits from symbiosis). Variation in nodule size was driven more by total nodule number than total nodule dry mass, such that the hosts forming the largest nodules with the most effective strain also formed relatively few nodules in total, potentially reflecting greater host control over the infection process. During co-inoculations, UCR and BMR hosts (but not Anz hosts) again formed larger nodules than other hosts and gained significantly more growth benefit than expected from the mean of single-inoculation treatments. This suggests that variation in host investment into nodules can influence host benefits even in a co-inoculation setting in which sanctions are invariant.

The drivers of variation in host investment are unclear. One possibility is that variation in host investment is driven by underlying variation in the magnitude of the benefit hosts gain from effective strains, which could create an appearance of host investment variation. However, Cla hosts gained an extraordinarily high amount of benefit from strains #18 and #38 (Fig. 2) and still displayed 'unscaled investment' in terms of nodule size. Alternatively, the ability to differentially invest in symbionts based on effectiveness could be costly for hosts (Foster & Kokko, 2006; Steidinger & Bever, 2014), similar to the observation that R-gene-mediated plant defense against pathogens can reduce the growth of disease-free plants (Tian *et al.*, 2003). We found that uninoculated UCR and BMR hosts, which displayed 'scaled investment', tended to have lower total plant dry mass than most 'unscaled investment' hosts, consistent with constitutive costs of host control (Table S5). However, Cla hosts also had relatively low plant dry mass and still displayed 'unscaled investment'. Furthermore, as ineffective strain #2 had greater population density in nodules compared to effective strain #18, similar-sized nodules occupied by different strains could still generate different fitness outcomes for the two strains. Thus, the drivers of variation in host investment into symbionts, and how this influences symbiont fitness in the soil, both merit further study.

Resource satiation hypothesis

Nitrogen fertilization has long been associated with reduced nodulation and biological N fixation in the agricultural sciences (Herridge & Rose, 2000; Van Kessel & Hartley, 2000; Wissuwa *et al.*, 2009). The energetic costs of building nodules and fueling the reduction of atmospheric N seem to provide an advantage to plants that exclusively use mineral sources of nutrients when they are plentiful. However, we did not find evidence for the resource satiation hypothesis in *A. strigosus*. Host sanctions were severe in both unfertilized and fertilized conditions, consistent with previous tests of sanctions (Kiers *et al.*, 2006; Regus *et al.*, 2014). Host investment during single inoculations was unaffected by fertilization for the two effective strains but decreased with fertilization for ineffective strain #2, suggesting that fertilization improved host control. A previous study of *A. strigosus* from BMR and UCR found that fertilization only reduced nodulation at levels that also caused high plant mortality (i.e. $>3.0\,g\,l^{-1}$ KNO_3, compared with $0.5\,g\,l^{-1}$ KNO_3 used here; Regus *et al.*, 2017). The fertilization-induced decline in strain #2 nodule size occurred well below the fertilization rate that causes host toxicity and probably represents adaptive host control. Furthermore, variation in host investment was not structured by the soil N regimes associated with those host populations, as the UCR and BMR hosts that displayed 'scaled investment' were from very high and low soil N regimes, respectively.

Our results contrast with findings from studies suggesting fertilization could erode host control. However, the best example of long-term N exposure reducing host control is confounded by crop breeding history, which generally does not target belowground traits and could allow host control traits to erode through drift (Kiers *et al.*, 2007). A long-term study suggested that N fertilization can reduce the effectiveness of rhizobia associating with wild *Trifolium* (Weese *et al.*, 2015), but hosts decreased in abundance during the study period, leading to fewer opportunities to interact with rhizobia and making it difficult to discern if hosts

also reduced their selection for symbiont effectiveness (i.e. the resource satiation hypothesis). Here, we found evidence that hosts maintain robust host control in fertilized conditions, consistent with the alternative hypothesis that plant fitness in high-N soil is maximized when hosts only permit the best symbionts to proliferate *in planta*, enabling the plant's modest N needs to be met with a minimum of cost to plant carbon (Kiers *et al.*, 2007). Thus, increased soil fertility may not contribute to the maintenance of variation in symbionts in natural systems, to the extent that symbiont effectiveness depends on host control traits as opposed to host ecology.

Partner mismatch and other hypotheses

Although we tested three models for the maintenance of variation in symbiont effectiveness, there are other hypotheses we did not test. For instance, ineffective symbionts may be primarily adapted to the free-living portion of their lifecycle (i.e. in soil between cycles of plant infection), which could eventually lead to mutualism abandonment (Denison & Kiers, 2004; Sachs & Simms, 2006). Consistent with the idea that symbionts can 'specialize' in the free-living portion of their bipartite lifecycle, some *Bradyrhizobium* genotypes exhibit greater metabolic flexibility than other symbiont genotypes (Hollowell *et al.*, 2016b) and are also epidemic in distribution across a metapopulation of symbionts (Hollowell *et al.*, 2016a). *In vitro* evolution further shows that without host interaction, rhizobia can rapidly erode in their symbiotic effectiveness on hosts (Sachs *et al.*, 2011b). Partner mismatch operating at a coarser host taxonomic scale could also maintain variation in symbiont effectiveness: there is evidence that ineffective strain #2 used in this study forms relatively large nodules on another host species, *A. wrangelianus* (Pahua *et al.*, 2018). Finally, a reasonable null model for the maintenance of symbiont variation is mutation-selection balance, whereby mutation events constantly generate variation in symbiont benefits, and the less effective genotypes are slowly purged from symbiont populations due to having lower-than-average fitness (Van Dyken *et al.*, 2011). Further work is needed to examine rhizobial fitness in hosts and soils to discriminate among these other hypotheses.

Conclusions

Here, we used three *Bradyrhizobium* strains and host lines from six *A. strigosus* populations to test for context dependency of host control, such that host control varies depending on availability of mineral N or the genotypes of the interacting partners. We found no evidence for the partner mismatch hypothesis, in which ineffective strains are maintained by being conditionally effective on other host genotypes. Instead, we found broad conservation of strain symbiotic effectiveness on hosts from across California. We found no evidence for the resource satiation hypothesis, in which hosts encountering high-N soils relax host control traits. Instead, we found that hosts significantly reduced investment into nodules occupied by the ineffective strain when they were fertilized, and co-inoculated hosts sanctioned the ineffective strain equally well in unfertilized and fertilized conditions, consistent with host control. Our data support the host variation hypothesis, in which hosts vary genetically in host control and thus vary in the selection they impose on symbiont effectiveness. Host sanctions against ineffective symbionts were robust in hosts from all populations, but we found variation in host ability to preferentially invest in nodule size according to symbiont effectiveness, even when plants were also enacting sanctions (i.e., in the co-inoculation experiment). This study contributes to reports of variation in host control from two other legume species (soybean, Kiers *et al.*, 2007; *Medicago lupulina*, Simonsen & Stinchcombe, 2014), suggesting that this could be a consistent feature of legume species that engage in symbiosis. Differences in symbiont fitness produced by the combined action of invariant sanctions and variable investment could help maintain variation in the effectiveness of symbiont populations.

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Author contributions

C.E.W., J.U.R. and J.L.S. planned and designed the research. C.E.W., J.U.R., K.A.G-C., A.C.H., K.W.Q., J.Y.L. and E.S.A. performed the experiment and collected data. C.E.W. and J.L.S. wrote the manuscript.

ORCID

Camille E. Wendlandt http://orcid.org/0000-0001-7901-3442
Kenjiro W. Quides http://orcid.org/0000-0003-2015-5264
Joel L. Sachs http://orcid.org/0000-0002-0221-9247

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Host symbiotic efficiency of *A. strigosus* in each treatment.

Fig. S2 Regression of rhizobial cells per nodule against nodule area.

Fig. S3 Total nodule dry mass and total nodule number of *A. strigosus* in each treatment.

Table S1 Collection information for *A. strigosus* host lines

Table S2 Genetic distance matrix of *A. strigosus* host lines at *nrITS* and *CNGC5* loci

Table S3 Statistical model selection

Table S4 Leaf %N and ^{15}N content of *A. strigosus* during single inoculations

Table S5 Total plant dry mass of *A. strigosus* in each treatment

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